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Immunocytochemical investigation of catalase and peroxisomal lipid β-oxidation enzymes in human hepatocellular tumors and liver cirrhosis

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Abstract A significant reduction of catalase activity, a peroxisomal marker enzyme, occurs in human hepatic neoplasias, but no information is available on other peroxisomal proteins. We have studied by means of immunohistochemistry four specific proteins of peroxisomes (catalase and three enzymes of lipid β-oxidation) in human hepatocellular tumors of various differentiation grades from adenoma to anaplastic carcinoma. In all tumors, except the adenomas, the tumor cells contained fewer peroxisomes than extrafocal hepatocytes and the reduction of antigenic sites in the tumor types generally correlated with the degree of tumor dedifferentiation as assessed by classical histopathological criteria. Two poorly differentiated tumors had no detectable peroxisomes at all. There were no major differences in the intensities of the immunocytochemical staining for all four studied peroxisomal antigens in different tumors, suggesting that the neoplastic transformation affects the biogenesis of the entire organelle and not merely the individual peroxisomal enzyme proteins. Some tumors exhibited a distinct peripheral distribution of peroxisomes. In cases with associated liver cirrhosis, the hepatocytes in the adjacent liver showed marked peroxisome proliferation, forming large perinuclear aggregates, occupying occasionally the entire cytoplasm. Taken together, our observations indicate that peroxisomes are significantly altered in both hepatocellular tumors and liver cirrhosis and, thus, could be responsible for some of the metabolic derangements observed in those disease processes.

Key words Peroxisomes · Hepatocellular tumors · Immunocytochemistry · Human

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Introduction

Ever since the report of Blumenthal and Brahn in 1910 [5] and the pioneering studies of Greenstein [14], it has been well known that catalase is significantly reduced in the liver of animals with neoplasia [15, 25]. Since the bulk of the catalase activity in the mammalian liver is localized in peroxisomes [11, 13], marked alterations of this organelle in conjunction with neoplastic transformation of the liver would be expected. The results of routine electron microscopical (EM) studies, however, have been somewhat controversial. While some authors found only a few microbodies in hepatic tumors [6, 36], others reported numerous profiles of peroxisomes and peroxisome-like structures in hepatocellular carcinomas (HCCs) [26, 29, 32]. The identification of peroxisomes in routine EM preparations, particularly in the absence of crystalline cores of urate oxidase – as observed in the human liver – however, can be difficult, and the results of such studies without proper sampling and morphometry can be highly biased. Roels and associates used catalase cytochemistry and EM morphometry analyzing the alterations of peroxisomes in the livers of patients with extra-hepatic tumors [9, 28]. In most cases, a focal perinuclear accumulation of smaller peroxisomes was reported. Their patient material also included two cases of hepatoma, one of which exhibited a similar peroxisomal alteration. In another case report of a patient with ‘nodular regenerative hyperplasia’, which is a benign hepatocellular tumor-like condition, a peculiar peripheral subendothelial distribution of peroxisomes was described [10].

Surprisingly, except for the above-mentioned few cases, there have been no reports on abundance and distribution of peroxisomes in human HCCs, using more

advanced cytochemical or immunocytochemical techniques. In an attempt to address this problem, we applied a method developed in our laboratory for the immunocytochemical demonstration of peroxisomal enzymes [18] and a modification of it for routinely processed, i.e., formalin-fixed and paraffin-embedded, human liver [19] to archival material. In the present study, antibodies to catalase and three enzymes of the peroxisomal lipid β-oxidation pathway have been applied to sections of selected cases of hepatocellular tumors exhibiting different grades of malignancy and different histologic patterns. In addition, the alterations of peroxisomes in the adjacent cirrhotic human liver tissue are reported.

Material and methods

Tissues

Samples of 12 hepatocellular tumors were obtained from the tissue archives of the Department of Pathology, University of Heidelberg. They were selected on the basis of histological grading and classification of primary hepatic tumors according to Edmondson and Steiner [12], and consisted of: (a) hepatocellular adenomas, (b) trabecular, pseudoglandular HCC II, (c) trabecular, clear cell partially pseudoglandular HCC II, (d) trabecular, mostly clear cell HCC II, (e) solid, partially clear cell HCC II/III, (f) trabecular HCC II/III, (g) trabecular HCC III and (h) giant cell anaplastic HCC IV. All samples were from patients undergoing partial hepatectomy for resection of histopathologically confirmed primary hepatocellular tumors. In some cases there was advanced cirrhosis in the adjacent liver.

Tissue processing

Small pieces of liver tumors with adjacent nontumorous hepatic parenchyma were excised, fixed for 24 h in 10% neutral, phosphate-buffered formalin and routinely embedded in paraffin.

Antisera

Polyclonal, monospecific antisera against guinea-pig liver peroxisomal catalase and rat liver peroxisomal enzymes of lipid β-oxidation: acyl-CoA oxidase, bi(multi)functional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/Δ^3,Δ^2-enoyl-CoA isomerase) and 3-ketoacyl-CoA thiolase were raised in rabbits and purified by means of affinity chromatography. Their specificity was confirmed by Western blotting, and their cross-reactivity with the corresponding human peroxisomal enzyme proteins was shown in our previous studies [18, 19].

Immunocytochemical technique

Peroxisomal proteins were detected in 4-µm paraffin sections using the indirect immunoperoxidase technique according to the procedure described previously [19]. Briefly, sections were deparaffinized in xylene, rapidly rehydrated via two changes of acetone and brought to phosphate-buffered saline (PBS). After blocking the endogenous peroxidase by H_2O_2-methanol, sections were treated with 1% bovine serum albumin (BSA) in PBS for 60 min, and were incubated overnight with the specific antisera to peroxisomal enzymes diluted 1:500–1:1000 with PBS (final antibody concentration 10–20 µg protein/ml). Following a wash with PBS, sections were incubated for 30 min with peroxidase-labeled swine anti-rabbit IgG (Dako) and the peroxidase activity was visualized using the 3-amino,9-ethylcarbazole (AEC) medium. Protease treatment of sections [19] was unnecessary in the investigated material. For optimal results, the final color development with AEC was extended to 60 min.

Control sections were incubated either with non-immune rabbit serum instead of the specific antibody, or directly in the AEC medium omitting the antibody step.

Parallel sections were stained with hematoxylin and eosin in order to correlate the histological patterns of the investigated tumors with the immunocytochemical results.

Results

Incubation of sections with all four antisera revealed a distinctly positive granular staining pattern, corresponding to the distribution pattern of peroxisomes as reported previously [18, 19]. They were present both in most tumor tissues and in the surrounding liver parenchyma. The specificity of the immunostaining was confirmed by negative results of control incubations either with non-immune serum or with aminoethylcarbazole alone. The antiserum against catalase yielded the strongest immunostaining, although in general there were no substantial differences in the intensity of peroxisomal staining after incubation with the different antisera. However, the abundance and the distribution patterns of immunostained peroxisomes present in cells of different tumors as well as in the surrounding liver tissue exhibited considerable variations as presented below. The occurrence of peroxisomes in tumors was histologically graded as (a) abundant (large), (b) mod-

Table 1 Quantity and distribution of peroxisomes in hepatocellular tumors. *HCC* hepatocellular carcinoma

Tumor type	Quantity of peroxisomes	Distribution of peroxisomes
Hepatocellular adenoma	Large (abundant)	Uniform
Trabecular, pseudoglandular HCC II	Moderate	At the periphery of pseudoacini (basal regions of the cells)
Trabecular, pseudoglandular, partially clear cell HCC II	Moderate in dark cells, low in clear cells	Uniform in dark cells, peripheral in clear cells
Trabecular, mostly clear cell HCC II	Moderate both in clear and dark cells	Peripheral in clear cells, uniform in dark cells
Trabecular HCC II/III	Very low	Solitary, occasionally in small clusters
Trabecular HCC III	Low	Variable, peripheral in “rosettes”
Solid, partially clear cell HCC II/III	Peroxisomes absent	–
Giant cell anaplastic HCC IV	Peroxisomes absent	–

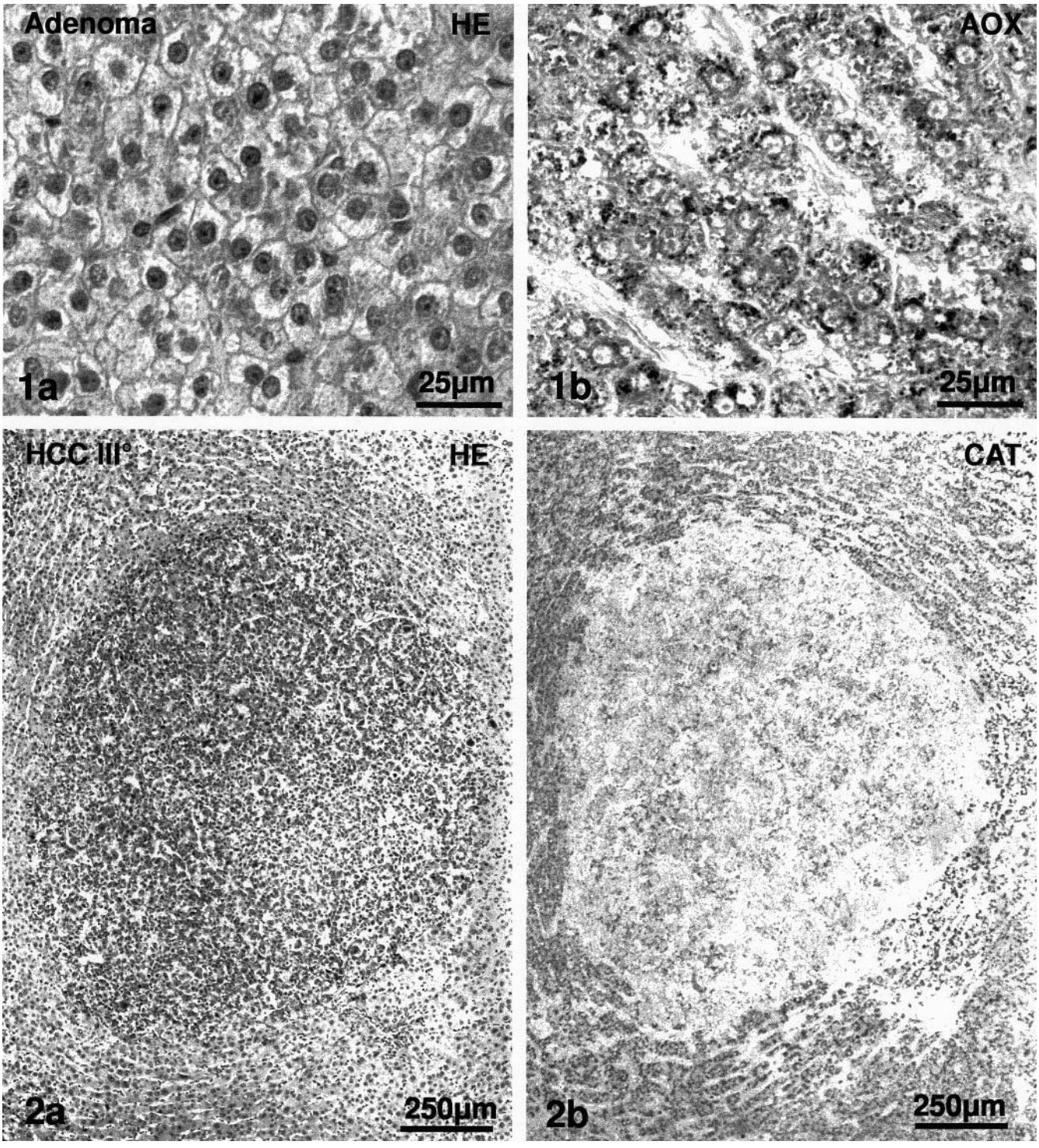


Fig. 1 Hepatocellular adenoma – **a** hematoxylin and eosin, **b** immunostaining for peroxisomal acyl-CoA oxidase (AOX). Most tumor cells contain abundant peroxisomes, quite uniformly distributed in the cytoplasm. A few cells show a perinuclear accumulation of peroxisomes or focal peroxisomal aggregates. ×500

Fig. 2 Hepatocellular carcinoma (*HCC*) III – **a** hematoxylin and eosin, **b** immunostaining for catalase. A small neoplastic nodule surrounded by hepatic parenchyma. Note the much weaker immunolabeling in the nodule due to a much lower frequency of peroxisomes in tumor cells than in the adjacent liver tissue. ×125

Fig. 3 Trabecular hepatocellular carcinoma (HCC) II – **a** hematoxylin and eosin, **b** immunostaining for catalase. A borderzone between the tumor (*T*) in upper part and the adjacent hepatic parenchyma (*N*) in lower part is depicted. Note the relatively strong immunolabeling for catalase in this well differentiated tumor. ×310 ▶

Fig. 4 Trabecular hepatocellular carcinoma (HCC) II/III – **a** hematoxylin and eosin, **b** immunostaining for catalase. A similar arrangement of tumor (*T*) and the adjacent liver (*N*) as in Fig. 3. Note the much weaker immunolabeling in this less differentiated tumor. ×310

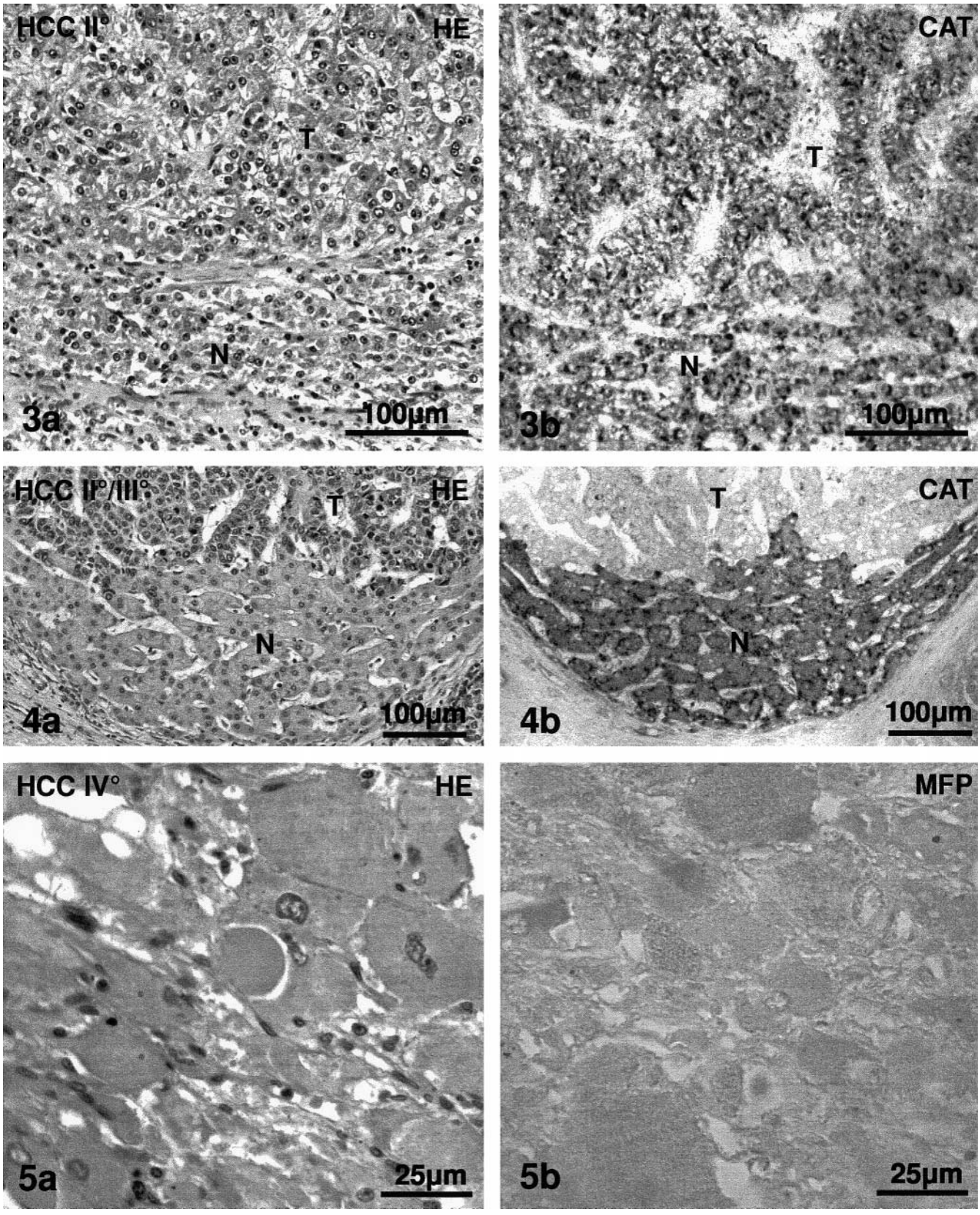


Fig. 5 Giant cell anaplastic hepatocellular carcinoma (HCC) IV – **a** hematoxylin and eosin, **b** immunostaining for bi(multi)functional enzyme (*MFP*) (hydratase-dehydrogenase-isomerase). The cytoplasm of tumor cells does not contain any immunoreactive peroxisomes. Similar results were obtained with all other antibodies tested. ×500

erate and **c** low with the first group showing a uniform prominent granular staining, the second containing focal scattered granules and the last one with only rare particles.

Tumor tissue

The quantity and distribution of peroxisomes present in the cells of the investigated liver tumors are summarized in Table 1.

In hepatocellular adenomas (Fig. 1), peroxisomes were abundant and their frequency and cytoplasmic distribution resembled that in normal liver [19]. Occasionally, however, some cells exhibited a perinuclear accumulation or large focal cytoplasmic aggregates of peroxisomes (Fig. 1b).

In HCCs (Fig. 2a), the intensity of immunostaining was clearly reduced relative to the adjacent non-tumorous hepatic parenchyma (Fig. 2b). The reduction of immunostaining, however, varied depending on the tumor type and grade, and showed even some variability within the same tumor. In most HCCs, the cells located at the periphery of the neoplastic nodules contained more peroxisomes than cells occupying their central areas. The intensity of immunolabeling and the number of peroxisomes ranged from moderate (in all tumors classified as grade II) (Fig. 3) to very low (Fig. 4). In two tumors (one solid HCC II/III and one giant cell anaplastic HCC IV) no immunoreactivity could be found at all (Fig. 5).

Tumor cells containing moderate to low amounts of peroxisomes showed various patterns of peroxisome distribution. In the pseudoglandular HCC (Fig. 6a), peroxisomes were located in a relatively narrow peripheral zone of the pseudoacini, i.e., in the basal regions of the cells (Fig. 6b). A special distribution was also observed in some areas of the trabecular HCC III, where cells are arranged in multilayered rosettes (Fig. 7a). In such foci, only cells located at the periphery of the rosettes contained peroxisomes, whereas those in the central zone did not (Fig. 7b). In clear cells present in several tumors (Fig. 8a), peroxisomes were consistently localized in cell periphery (Fig. 8b) in contrast to dark cells, which, even when coexisting with the clear cells in the same tumor, exhibited a more uniform distribution of these organelles. In tumors with very few peroxisomes, these were present only in a small fraction of cells either as large solitary particles or as small clusters. One of those tumors, a trabecular HCC II/III, had a few areas with cells showing the presence of very large (up to 6 μm) immunoreactive granules (Fig. 9), which were positive with all antibodies tested.

Cirrhotic liver tissue

The liver tissue surrounding the tumors in some cases showed evidence of moderate to advanced hepatic cirrhosis. The immunostained peroxisomes of hepatocytes in such areas also exhibited considerable abnormalities dependent on the severity of the pathologic process in the hepatic tissue. In two cases with only mild fibrosis of the liver, the hepatocellular peroxisomes appeared nearly normal [19] as far as both their quantity and distribution were concerned, with only some cells exhibiting perinuclear aggregates of peroxisomes (Fig. 10).

In cirrhotic liver tissue, however, hepatocytes contained clearly an increased number of peroxisomes, very often occupying the perinuclear area. In most hepatocytes peroxisomal aggregates filled up large parts of the cytoplasm (Fig. 11). In some cells the entire cytoplasm was densely packed with peroxisomes (Fig. 12). Infrequent binuclear hepatocytes also showed accumulations of peroxisomes either surrounding the nuclei or located at the two opposite poles of the cell (Fig. 13). An interesting, albeit rare observation was a giant polyploid hepatocyte containing two large conical aggregates of peroxisomes flanking the nucleus (Fig. 14).

All control preparations were clearly negative confirming the specificity of the antibodies and the reliability of the immunohistochemical method used.

Discussion

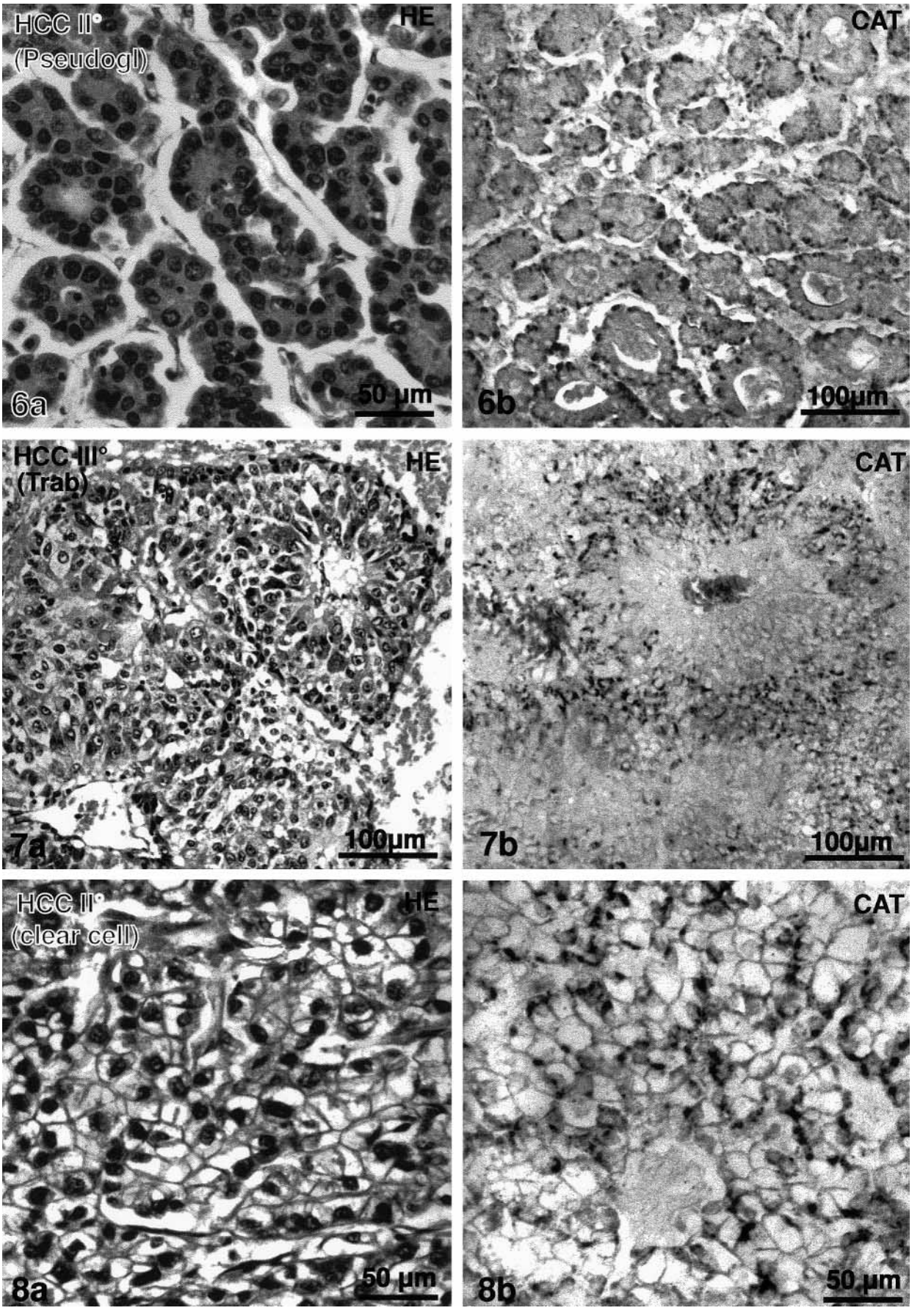
Although the peroxisomes in human HCC have been extensively studied by EM [6, 9, 10, 26, 28, 29, 32, 36], this is the first report combining the specificity of the immunolabeling with the advantages of sampling large sections by light microscopy for the investigation of their alterations. Thus, the abundance and the distribution of peroxisomes are clearly visualized in HCC (Fig. 2b) without the sampling problems of routine EM and the hardships of ultrastructural morphometry.

An important observation of our study was that all four peroxisomal proteins studied (catalase and three enzymes of peroxisomal lipid β-oxidation) were affected similarly in all the tumors analyzed, suggesting that the biogenesis of the whole organelle may be altered in the process of neoplastic transformation. This is in full agreement with the recent observations of Yokoyama et al. [37] on reductions of several peroxisomal enzymes in preneoplastic nodules and hepatomas in rat liver induced by prolonged treatment with peroxisome-proliferators [27]. Moreover, very recent findings from our laboratory suggest that indeed the biogenesis of peroxisomes may be impaired also in human adenocarcinomas of colon [17].

Fig. 6 Pseudoglandular hepatocellular carcinoma (HCC) II – **a** hematoxylin and eosin, **b** immunostaining for catalase (*CAT*). Peroxisomes are located almost exclusively at the periphery of pseudoacini, i.e., in the basal regions of the tumor cells. ×310 ▶

Fig. 7 Trabecular hepatocellular carcinoma (HCC) III – **a** hematoxylin and eosin, **b** immunostaining for catalase (CAT). In rosette-like formations, peroxisomes occur mostly in the peripheral cells but not in the central ones. ×310

Fig. 8 Clear cell hepatocellular carcinoma (HCC) II – **a** hematoxylin and eosin, **b** immunostaining for catalase (CAT). Note a peripheral localization of immunostained peroxisomes in clear-type tumor cells. ×500


HCC II°
(Pseudogl)
HE
6a
50 µm
CAT
6b
100µm
HCC III°
(Trab)
HE
7a
100µm
CAT
7b
100µm
HCC II°
(clear cell)
HE
8a
50 µm
CAT
8b
50 µm

The abundance of peroxisomes in HCC correlates with their degree of differentiation

The frequency of peroxisomes in human hepatocellular tumors seemed to correlate well with their degree of differentiation (Table 1). Thus, in hepatocellular adenomas and well differentiated HCC II, immunolabeling revealed abundant peroxisomes, while, in anaplastic carcinoma IV and solid carcinomas III, all antibodies against peroxisomal proteins gave negative results. This finding is consistent with the observations in experimental liver tumors in rodents induced by different hepatocarcinogens [20, 22, 23, 24] and indicates that an inverse relationship exists between the tumor growth rate and the abundance of peroxisomes.

Since the completion of this study and after its submission for publication, Suto et al. [34] also reported a significant reduction of the peroxisomal bi(multi)functional enzyme in low grade human HCCs (grades II–IV). In addition, they showed, by means of Western blotting and enzyme activity measurements in homogenates of selected tumors, the loss of protein and enzyme activity in less differentiated lesions.

Recently, Bannasch and co-workers compared the expressions of key enzymes of energy metabolism in two distinct types of preneoplastic foci in rat liver: the 'glycogen storage foci' induced by *N*-nitrosomorpholine and the 'amphophilic cell foci' generated by treatment with dehydroepiandroesterone [22, 23]. Interestingly, the 'glycogen-rich foci' contained much fewer peroxisomes than the 'amphophilic cell foci', which showed even focal increases of peroxisomal hydratase and acyl-CoA oxidase [22]. This is of great interest because HCCs, which arise from the latter lesions, are more differentiated than those developing from the former, thus confirming that the abundance of peroxisomes even in preneoplastic foci correlates well with their differentiation.

The exact mechanisms underlying the reduction of peroxisomal enzymes in HCC are not known, but Sato et al. [31] reported a significant downregulation of transcription of catalase gene in rat and human hepatoma cells. Those authors identified also the core sequence of a 'silencer element' in the catalase gene and a 35-kDa nuclear protein as its likely ligand, which was present only in hepatoma cells but not in the normal liver.

The cytokine, tumor necrosis factor-α (TNF-α) is another likely candidate that could contribute to the downregulation of peroxisomal proteins. We have shown recently that intravenous injection of recombinant TNF-α reduced significantly the mRNA levels of several peroxisomal proteins [3]. A similar reduction of corresponding proteins was also reported previously [2]. Interestingly, we have also found a TNF-α-dependent reduction of peroxisome proliferator activated receptor (PPAR-α), both at the protein and mRNA levels [3]. Further studies of this nuclear transcription factor and its related regulatory proteins in HCC should be useful in elucidating the mechanisms of downregulation of peroxisomal proteins in hepatic tumors.

Peroxisomes exhibit distinct patterns of intracellular distribution in some HCC

Whereas in normal hepatocytes and well-differentiated hepatomas peroxisomes are uniformly distributed in the cytoplasm (Fig. 1b), in some histologic types, such as the pseudoglandular HCC, they are confined to the basal portions of tumor cells (Fig. 6b). In clear cell carcinomas peroxisomes were also found at the cell periphery (Fig. 8b). Whereas in the latter tumors the large amounts of glycogen in the cytoplasm could be responsible for the peripheral displacement of organelles, in the former tumors some other mechanisms might be involved. A similar localization of peroxisomes beneath the sinusoidal surface of hepatocytes was also reported in a patient with 'nodular regenerative hyperplasia' of the liver [10]. We have also noted in the normal guinea-pig hepatocytes a peripheral subsinusoidal accumulation of peroxisomes and have suggested that this may be related to the high levels of circulating chylomicrons and plasma etherphospholipids in this species [21]. In patients with HCC, abnormalities of the lipid metabolism with hypercholesterolemia has been well known [1], and this seems to be due to the loss of feedback control in the biosynthesis of cholesterol in hepatoma cells [4]. Since peroxisomes are involved in the biosynthesis of cholesterol [16], further studies on the alterations of peroxisomal HMG-CoA reductase and its regulation in HCC would be of great interest.

Massive proliferation of peroxisomes in adjacent cirrhotic liver tissue

Although the main objective of this study was the assessment of peroxisomal alterations in human HCC, we found a massive proliferation of peroxisomes in the adjacent non-tumorous liver tissues exhibiting cirrhosis. The severity of peroxisome proliferation, in contrast to the

Fig. 9 Trabecular hepatocellular carcinoma (HCC) II/III immunostained for catalase (CAT). Some cells in this less differentiated tumor contain very large immunoreactive granules (*arrows*), while others have very few or none. ×500 ▶

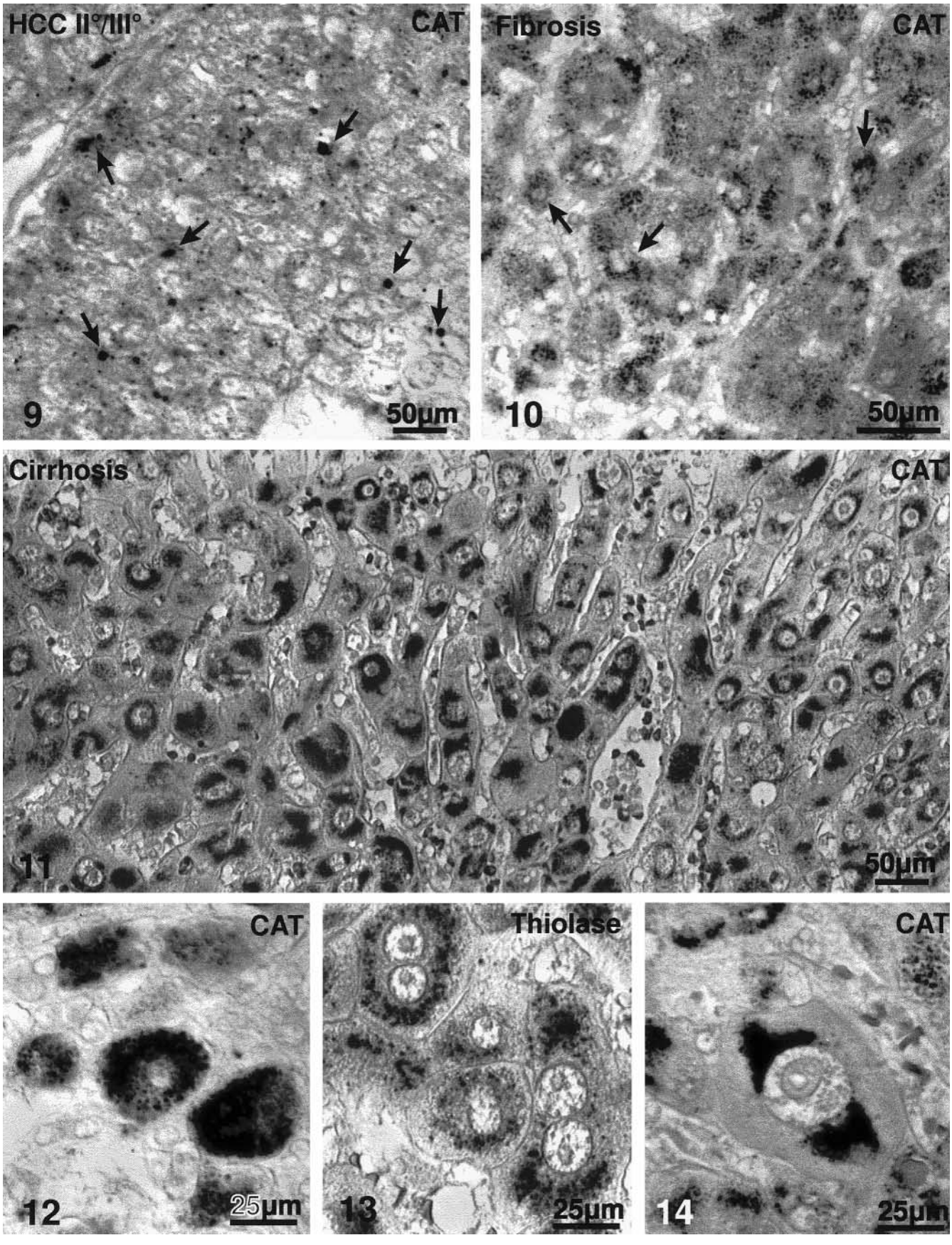
Fig. 10 Liver parenchyma with relatively mild fibrosis, immunostained for catalase (CAT). Most hepatocytes show an almost normal quantity and distribution of peroxisomes with a few cells exhibiting perinuclear aggregates (*arrows*). ×500

Fig. 11 Severely cirrhotic liver. Note the marked proliferation of peroxisomes and the altered pattern of peroxisome distribution with large aggregates occupying a substantial portion of the cell cytoplasm. Immunostaining for catalase (CAT). ×500

Fig. 12 Cirrhotic liver. Hepatocytes with their entire cytoplasm densely packed with peroxisomes. Immunostaining for catalase (CAT). ×800

Fig. 13 Cirrhotic liver. Two binuclear hepatocytes with peroxisomal aggregates surrounding the nuclei or flanking them. Immunostaining for thiolase. ×800

Fig. 14 Cirrhotic liver. A giant polyploid hepatocyte with two large peroxisomal aggregates flanking the nucleus. Immunostaining for catalase (CAT). ×800


HCC II°/III°
CAT
9
50µm
Fibrosis
CAT
10
50µm
Cirrhosis
CAT
11
50µm
CAT
12
25µm
Thiolase
13
25µm
CAT
14
25µm

reduction of peroxisomes in most tumors, was so impressive that we decided to include those observations in this paper (Fig. 10, Fig. 11, Fig. 12, Fig. 13, Fig. 14), particularly since there have been no reports of immunohistochemical investigation of peroxisomes in cirrhosis. Indeed, by means of light microscopy and immunohistochemistry in paraffin sections, the severity of peroxisome proliferation, occupying almost the entire cytoplasm of some hepatocytes, could be visualized for the first time (Fig. 11 and Fig. 12). In previous EM studies of human liver cirrhosis, no changes of peroxisomal volume density was noted [30], although a numerical increase of small peroxisomes with focal perinuclear accumulation was found by EM morphometry [8]. The intensity of DAB-staining for catalase was reported to be decreased in most cases [8, 28]. The proliferation of peroxisomes in cirrhotic liver is not unexpected since a participation of catalase in metabolism of ethanol has been well known [35] and a proliferation of peroxisomes has been reported also in cases of alcoholic hepatitis [7]. Further clinical pathological studies using immunohistochemistry are needed to further elucidate the alterations of peroxisomal proteins in the developmental stages of hepatic cirrhosis, particularly since hitherto only the alterations of catalase have been reported [8, 28].

A focal perinuclear aggregation of peroxisomes was observed in this study not only in hepatic fibrosis (Fig. 10) and cirrhosis (Fig. 11, Fig. 12, Fig. 13, Fig. 14), but also in adenomas (Fig. 1b) and this is generally considered to be due to proliferation of peroxisomes [8, 28]. We found recently that depolymerization of the microtubular system in human hepatoblastoma cells HepG2 induces the formation of large cytoplasmic aggregates of peroxisomes, which rapidly disappear once the microtubules are allowed to regenerate [33]. Thus, the perinuclear accumulation of peroxisomes could be a reflection of such alterations of the microtubular network, justifying further studies of the cytoskeleton under the above-mentioned conditions.

In conclusion, the present immunohistochemical study has revealed that peroxisomes in human liver are markedly altered in HCC and in cirrhosis. Whereas in hepatic tumors a reduction of peroxisomes is observed to be dependent on the degree of dedifferentiation of hepatocytes and the grading of HCC, in cirrhosis in adjacent liver tissue a significant proliferation of peroxisomes is observed.

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